INHIBITION OF RUMINAL UREASE *IN VIVO* BY ACETOHYDROXAMIC ACID

by

FABIO de BORJA PORTELA

B. S., University of Minas Gerais, Brazil, 1963

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Animal Husbandry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1968

Approved by:

Major Professor

ACKNOWLEDGMENTS

I sincerely express my appreciation to Dr. B. E. Brent, my Major Professor, for his guidance in planning, advice in the organization and general conduct of this research. Thanks to his cooperation it was possible for me to conclude this work.

I am deeply grateful to the U. S. Aid and to IRI Research Institute for the financial support during the years of 1967-1968, the period during which the author stayed in the United States.

My sincere thanks to Dr. Robert Schalles for statistical analysis and Amos Adepoju for help in conducting the research.

TABLE OF CONTENTS

INTRODUCTION

A number of non-protein nitrogen sources have been studied concerning their role in supporting microbial protein synthesis in the rumen. Of these compounds, urea has enjoyed the most extensive investigation and application.

Rumen microorganisms produce a large amount of urease, an enzyme which rapidly catalyzes the hydrolysis of urea to ammonia. The ammonia thus released is used by the rumen microorganisms in the synthesis of their own protoplasm. Unfortunately, the resulting high level of ammonia often exceeds the capacity of protein synthesis by the rumen microorganisms, and it is absorbed through the rumen wall into the venous blood draining the rumen. It is transformed into urea in the liver and is almost completely excreted through the kidneys. For optimum nitrogen utilization, ammonia levels in the rumen should be adequate to support maximum protein synthesis by rumen microorganisms and at the same time, low enough to prevent an excessive loss of ammonia from the rumen into the circulation. The high urease activity of the rumen content causes very rapid urea hydrolysis and results in excessive rumen ammonia levels within a short time after feeding.

Inhibition of the enzyme, urease, has been proposed as a method of reducing these high ammonia levels. Several compounds have been examined as urease inhibitors. Loper (1967) studied several metal ions and antibiotics. Harbers *et al.*,

(1962) examined the inhibition of urease with barbituric acid. The non-specific nature of these compounds has made their use in the rumen impractical.

Acetohydroxamic acid has been proposed as a means of preventing ammonia toxicity in uremic humans (Fishbein *et al.*, 1965). Brent and Adepoju (1967) have shown this compound to inhibit urease and decrease ammonia levels *in vitro* and *in vivo*.

This study was designed to more closely examine the effects of acetohydroxamic acid on the rumen ammonia level and to ascertain whether or not acetohydroxamic acid yields residual effects on urease activity after withdrawal of the inhibitor from the diet.

REVIEW OF LITERATURE

Characteristics of Urease

Urease was the first enzyme to be isolated (Sumner, 1926). Later work by Sumner and Hand (1928) described how urease was purified and crystallized and observed that recrystallized urease was more potent. Gorin and Chin (1965) found urease to have a molecular weight of 480,000 with eight active sites.

Chemical Properties. Several authors (Gorin *et al.*, 1962; Seneca *et al.*, 1962) have shown urease to have a molecular weight of 473,000 and Michaelis Constant 0.003M (Harmon and Nieman, 1949). Urease apparently does not require a coenzyme (Sumner and Somers, 1953). Gorin *et al.*, (1962)

reported that the urease molecule contains about 77 methyonyl, 29 cystinyl, and 47 cysteinyl residues. They noted that while some of the -SH groups showed great activity, the -SH groups necessary to enzymatic activity were observed to be less reactive. Seneca et al., (1962) found that the urease molecule had a total of 37 -SH groups. They also pointed out that urease hydrolyzes urea through a mechanism involving the -SH groups of the enzyme. Some of the mercapto groups are very reactive and may be involved in exchange reactions with disulfide groups, causing polymerization. Hellerman et al., (1943) studying the protein sulphydryl group and the reversible inactivation of the enzyme urease, found evidence that there is a relationship between the role of certain sulphydryl groups and the control of urease, a sulphydryl enzyme. This work presents direct evidence that there are two kinds of -SH groups, only one being essential to the control of the enzyme activity.

Seneca et al., (1962) observed that the optimum pH for urease activity is between 7.2 and 7.5. Several authors have reported different values. Bahadur and Chandra (1960) reported values between 6 and 8 for soybean urease. Henderick (1962) found maximum activity of ruminal urease at a pH of 8.25.

Seneca et al., (1962) found that urease remained active up to a temperature of 70° C.

The substrate specificity of urease is very high. Its action has been tested on a large number of substituted ureas,

none of which appears to be attacked, with possible exceptions of the dimethyl- and diethyl-ureas (Baldwin, 1967). Fishbein (1967) also demonstrated that hydroxyurea and dihydroxyurea are substrates for urease.

Wang and Tarr (1955) proposed three mechanisms for the hydrolysis of urea by urease in slightly acid solution. First is the carbonic acid mechanism, in which urea is assumed to be hydrolyzed first to carbamic acid, then to carbonic acid, and finally to carbon dioxide and water; secondly there is the carbamic acid mechanism in which urea is first hydrolyzed to carbamic acid and then the latter decomposes directly to carbon dioxide and ammonia without going through the carbonic acid stage. A third mechanism, in which urea is directly hydrolyzed to carbon dioxide and ammonia without going through the carbamic acid stage, is called the carbon dioxide mechanism. Only the carbamic acid mechanism is consistent with the experimental results. Bahadur and Chandra (1959), studying the decomposition in vivo of a 4% solution by urease at pH 6.7, 7.8, 8.8, 9.8, and 10.8, concluded that the optimum pH was 8.8. Rate of urea hydrolysis increased with increasing concentration of the urease. Perlzweig (1932), observed that heavy metals "poison" urease whereas cyanides, sulphydric acid, and certain amino-acids "probe" the enzyme or activate it after it had been "poisoned." He suggested that the thiol groups are not solely responsible for urease activity because urease solutions oxidized by aeration or by dihydrogen peroxide

to complete loss of activity can be reactivated, but to a very slight extent, by means of -SH compounds, sulfides or cyanides. Gorin et al., (1962) observed the participation of thiol groups in polymerization reactions. Fasman and Nieman (1951) investigated the factors governing the kinetics of the urease catalyzed hydrolysis of urea in aqueous solutions buffered at a pH of 7.0 with sodium and potassium phosphate. Both of the buffer components participated in the hydrolytic reaction. The buffer anion apparently functioned as an enzyme activator and the buffer cation as an inhibitor.

Occurrence of Urease. Urease is widely distributed in nature, occurring in large concentration in certain seeds, particularly jackbean and soybean. It is found in certain plant tissues, in the tissues of a few invertebrates, and in bacteria (Larson and Kallio, 1954). It has not been found in vertebrate tissues (Baldwin, 1967).

Bacterial Urease. Larson and Kallio (1954) purified, but were unable to crystallize urease from Bacillus pasteurii. Their preparation had the same characteristics as urease extracted from jackbeans by Sumner (1926). Muhrer and Carrol (1964) showed that the capacity of rumen microorganisms to hydrolyze urea exceeds their capacity to metabolize ammonia. They also showed that isolated ureolytic microorganisms from a fistulated sheep and steer showed a low level of urease activity. Thus, urease activity apparently occurs in many rumen species rather than a few highly active urea hydrolyzers.

Akkada and Blackburn (1963) reported similar conclusions. McKay and Oxford (1954) isolated from the rumen of calves an unidentified, small catalase-negative, Gram-negative rod which readily produced ammonia from urea. Gibbons and Doetsch, (1959) exploring the microflora of bovine rumen, isolated and characterized an organism related to Lactobacillus bifidus, a ureolytic type.

The majority of available data (Lattimer et al., 1961; Seneca et al., 1962) shows that bacterial urease is an intracellular enzyme. Jones et al., (1964), measuring the urease activity of strained rumen fluid from fed animals, showed that intracellular bacterial urease was responsible for ureolysis by rumen contents. When the rumen microflora was subjected to differential centrifugation, 65% of the total urease activity was found associated with a group of larger microorganisms which sedimented at 1200g. An average of 35% of the total viable bacteria in the rumen content were found by a dilution count technique to manifest urease activity. Brent and Richardson (1967) used centrifugation and sonic disruption on rumen microbes. Their data supported the intracellular nature of urease. Seneca et al., (1961) observed that sonic lysates of "urease negative" pathogenic bacteria become highly active in splitting urea after cell lysis, indicating that these bacteria also contain urease within the cells, but either the enzyme, the substrate or the product cannot diffuse through the cell wall. Lyubimov (1955)

concluded that urease elaborated by Micrococcus urae and Proteus vulgaris cultures is mainly formed during aging and disintegrating of the cell. The organisms do not elaborate urease into the surrounding medium. It is only during massive lysis that slight urease activity appears in the filtrate from the cultures. In living cells the breakdown of urea is an intracellular process. Levenson et al., (1959) concluded that enzymatic ureolysis in mammals is mediated by bacteria. Thus urease is not a constituent of mammals tissue. Fedorova (1965) examined the urease activity of several bacteria and concluded that bacteria contain three urease fractions. One is released in the course of growth. A second fraction is labile, bound by the cell surface, and is easily separated by washing. A third fraction is extracted only after disruption of the cells. Several authors (Shimura et al., 1958; Seneca et al., 1962); Gibbons and McCarthy, 1957); Gibbons and Doestch, 1959) also reported the intracellular origin of bacterial urease.

Although most workers have shown urease to be intracellular, Clifford et al., (1968) have found no difference in supernatant and sediment urease following centrifugation up to 25,700g. The discrepancy in these results must await further investigation.

Inhibition of Ruminal Urease. As shown by Muhrer and Carrol (1964) the rumen microorganisms are not capable of utilizing all the ammonia from urea hydrolysis. The

regulation of the enzymatic action upon urea is a major problem in the nutrition of ruminants. Several authors have studied the inhibition of urease with various compounds. Shaw (1953) studied the action of metal ions upon urease. The silver ion was found to be an extremely efficient inhibitor. Rumen urease has been inhibited by stannous chloride (Bahadur and Chandra, 1960) and by copper (Johnson et al., 1962). Harbers et al., (1962) studied the action of barbituric acid upon urease activity. Loper et al., (1967a, 1967b) studying urease inhibition in vitro and in vivo, found that copper, cobalt, and neomycin and bacitracin-MD, among several antibiotics tested are urease inhibitors.

Unfortunately the action of these compounds is not specific. They will also poison enzymes needed to carry out other necessary reactions.

Glimp and Tillman, (1965) studying the immunization of sheep against urease, found that ammonia levels in the ruminal vein were higher in control sheep than in those which had been immunized. Chapula et al., (1968a, 1968b) studying ammonia metabolism in the rumen and liver of ruminants, found that urease levels were higher in animals fed soy protein than in those receiving a diet containing urea. Since the urease levels may decrease during adaptation to urea, adapted animals may be less susceptible to urea toxicity.

Urease shows the peculiar property of being inhibited by a large quantity of its substrate. This effect may be

eliminated by the addition of glycine (Baldwin, 1967).

Loper, (1967) reviewing urease inhibitors, observed the possible use of hydroxamic acids. Kobashi et al., (1962) found that the alkyl and aryl derivatives of hydroxamic acids were highly specific and potent inhibitors of urease. These compounds were as inhibitory as acetohydroxamic acid, but salicylhydroxamic acid was a weaker inhibitor. They (Kobashi et al., 1962) also found that monohydroxamic acid of dicarboxilic derivatives generally showed little or moderate effect. The position of the basic group in the N-containing compound influenced the inhibition. Urease inactivated by acetohydroxamic acid was crystallized as an inactive complex which regained activity when treated one hour at 50° C at slightly alkaline pH in the presence of thioglycollate. Kobashi et al., (1966) reported that two molecules of hydroxamic acid bound one molecule of urease to form an inactive enzyme-inhibitor complex. From this they suggested that urease may have two active sites. They noted that most of the -SH groups are involved in the maintenance of the enzyme configuration rather than in direct function at the active site. Baintner (1964a) prevented urea poisoning in ruminants (sheep and goats) by inhibiting rumen urease with acetohydroxamic acid. When the urease was inhibited, urea was absorbed intact through the rumen wall and was less toxic than ammonia. Baintner (1964b) found that the optimum pH for rumen urease was 7.6, and that inhibition by acetohydroxamic acid appeared competitive. On

the other hand Fishbein and Carbone (1965) found urease inhibition by acetohydroxamic acid to be irreversible, complete and probably non-competitive. Ayoga and Summerskill (1966) found that acetohydroxamic acid inhibited 90% of fecal urease, and did not show toxicity. Pianotti et al., (1966) found that 1000 mcg./ml. of acetohydroxamic acid inhibited the ureolytic activity of intact cells in urea broth without concurrent growth inhibition. Hase and Kobashi (1967) noted that acetohydroxamic acid inhibition of extracts and intact cells of Proteus vulgaris was progressive with time. Caprylhydroxamic acid was the most potent inhibitor used by these workers. Inhibition of cell-free urease was not relieved by cysteine. Brent and Adepoju (1967) partially inhibited urease activity in intact steers with 3 gm. per feeding of acetohydroxamic acid.

Properties of Hydroxamic Acids

Chelating Properties. Emery and Neilands (1959) isolated ferric hydroxamates during the fermentation of Ustilago sphaerogena. The iron-coupling center appeared to be a trihydroxamic acid in a polydentate ligand of unknown structure. Abe (1960) reported that they have an ability to chelate ions. Coutts (1967) found that hydroxamic acids are strong chelating agents, forming insoluble complexes with many metals. Neilands (1967) also showed that these compounds are sophisticated ligands that play an important role in the metabolism

of microorganisms.

Fungistatic Activity. Alkiewicz et al., (1957) showed that in vitro, salicylhydroxamic acid, benzohydroxamic acid, 2-hydroxy-3-naphtohydroxamic acid and chloro-derivatives of phenoxyacetohydroxamic acid have strong fungistatic activity. Eckestein and Urbanski (1956) found some aryloacetohydroxamic acids to have fungicidal activity. Halweg and Krakouka (1956) studied the aromatic hydroxamic acids, benzo, salicyl, and 2-hydroxy-3-naphtohydroxamic acid and concluded that the fungistatic activity was attributable to the hydroxamic group. Ecstein and Czerwinskafejgin (1959) noted that aryloxy acetohydroxamic acid may or may not have fungicidal activity depending on the radical substitution. Arct et al., (1964) tested some aryloxyalkanehydroxamic acids in search of non-phytotoxic fungicides and concluded that some have fungicidal activity. Eckestein and Domanoka (1965) reported on the fungicidal activity of some derivatives of hydroxamic acids. Mostafa et al., (1966) observed fungicidal activity from some thiohydroxamic acid.

Bactericidal Activity. Abe (1960) showed that there is a relationship between anti-bacterial activity and chelating activity in cyclic hydroxamic acids. Coutts, (1967) in a review on acyclic hydroxamic acids, concluded that their antimicrobial activity is related to their ability to chelate metals and make them unavailable for the growth of microorganisms. Previous work by Coutts et al., (1965) with

quinolines containing a cyclic hydroxamic acid group had shown that other factors in addition to the presence of a hydroxamic acid group were necessary for anti-bacterial activity. Coutts and Hindsmarsh (1966) studying 2-H, 1-4-benzoxazine hydroxamic acid for its anti-bacterial activity on E. coli and S. aureus, concluded that it had activity at a concentration of 40 mg. per 100 ml. In other work Coutts et al., (1964) studying in vitro activity of certain quinolines, quinazolines, quinoxalines, and benzothiazine, showed that no compound had a broader spectrum of activity than 1-2-dihydro-1-hydroxy-2-oxoquinoline. Caradonna and Stein (1960) synthesized some hydroxamic acid derivatives of isoxazole and concluded that several had anti-bacteriostatic activity. Gale (1966) observed that oxamyl-hydroxamic acid inhibited the growth of E. coli by 50% in an 18-hour incubation and also inhibited the growth of Gram-negative organisms, including Pseudomonas, Proteus, Aerobacter, Alcaligenes, Paracolobactrum and Mycobacterium, but had no effect on Gram-positive bacteria. Gale and Hynes (1966) showed that glycylhydroxamic acid is an anti-bacterial agent. Noble and Wibberly (1966) showed that some quinoline-containing cyclic hydroxamic acids have anti-bacterial activity. Fishbein et al., (1965) reported that acetohydroxamic acid had no anti-bacterial activity against S. aureus, Streptococcus group A, E. coli, P. aeruginosa, Proteus mirabilis, and species of Brucella, Bordetella, and Klebsiela, and inhibited urease produced by these microorganisms with the exception of

streptococcus group A and E. coli.

Hydrolysis of Hydroxamic Acids. Bernheim (1964) studied the enzymatic hydrolysis of hydroxamic acids corresponding to the straight chain mono-carboxylic acids from valeric to decanoic, and monohydroxamate derivatives from malonic to adipic. He concluded from their hydrolysis in the presence of liver homogenates that at least two enzymes are present; a lipase hydrolyzing the straight-chain acids, and an enzyme that attacks succinyl and glutaryl monohydroxamates but not the corresponding malonyl and adipyl derivatives. The lipase acting on the hydroxamates of the monocarboxylic acids hydrolyzed those with a chain length from five to ten carbons. Bernheim (1965) observed that the hydroxamate corresponding to anthranilic acid disappeared when incubated with rat liver slices. The mechanism of the reduction of this acid was not established.

Toxicity of Hydroxamic Acids. Venulet et al., (1956) found the principal site of detoxification of salicylhydroxamic and 5-bromosalicyl acids to be the kidneys and the liver. Utley (1963) found that 1.0 mg./gm. of the rat body weight caused depression for 10 hours, and half of the dose caused tranquilization as defined by the suppression of the conditioned avoidance reaction. Epstein et al., (1956) measured the toxicity of 17 hydroxamic acid analogs and observed that it varied over a wide range. Baintner (1964a) found that acetohydroxamic acid had no toxic effect at doses of

0.25 gm./kg. for sheep and 0.44 gm./kg. for goats. Lambelin et al., (1966) found that p-butoxyacetohydroxamic acid demonstrated low acute toxicity, with LD_{50} values greater than 8 gm./kg. in the mouse and 4 gm./kg. in the rat. Chronic administration (500 mg./kg. of body weight daily) produced digestive tract lesions after one to two months. Doses of 250 to 500 mg./kg. diminished the growth rate due to lack of food utilization. Doses of 125 mg./kg. were well tolerated by animals. Fishbein et al., (1965a) found that acetohydroxamic acid in a single dose of 900 mg./kg. was toxic to rats. The full doses were recovered in the urine 24 hours following administration.

Absorption and Excretion of Hydroxamic Acids. Fishbein and Carbone (1963) concluded that acetohydroxamic acid is hydrolyzed by enzymes in vivo to free hydroxylamine. They also identified the intact acid in the urine of goats and sheep one hour after oral administration. However, they could not detect the compound in the blood of the animals. Roncucci et al., (1966) studying the distribution, metabolism and excretion of ^{14}C p-butoxyphenyl-acetohydroxamic acid in rabbits, found that after oral administration, reabsorption is rapid and represents 40 to 45% of the administered dose. The amounts recovered in the various organs are always less but parallel to amounts in the plasma, except in the kidneys and liver where it persists longer. A preferential localization is observed in the adrenal cortex. The radioactivity

recovered in the excreta represents 80% of the administered dose; 56% is found in the urine and 44% in the feces. Excretion was practically complete 24 hours after administration. Eleven metabolites were detected, among them, p-butoxyphenylacetic acid. Fishbein and Streeter, (1968) studying the physiological disposition of acetohydroxamic acid in the mouse using acetohydroxamate-1-^{14}C, concluded that this compound is rapidly but incompletely excreted in the urine, 60% appearing in the first 8 hours, and 10% in the next 16 hours, with negligible excretion during the second 24 hours. An additional 15% of the injected dose appeared in the first 24 hours in urine as ^{14}C-acetamide, identified by paper chromatography in 3 solvents. Only 1% of the injected compound appeared in the feces, and 6% was expired as $^{14}CO_2$. At 24 hours post-injection, total tissue content was about 2% of the injected compound and was evenly distributed in all organs studied. Maximum blood levels occurred within 2 hours after injection, and the rate of subsequent decline suggested distribution throughout total body water.

EXPERIMENTAL PROCEDURE

Preparation of Acetohydroxamic Acid. Acetohydroxamic acid was prepared according to Fishbein et al., (1965b), as follows. One mole of ethyl acetate was dripped slowly into a chilled (crushed ice) flask equipped with a magnetic stirrer and containing one mole of hydroxylamine hydrochloride

and 1.5 mole sodium hydroxide in 300 ml. of 30% ethanol. The mixture was kept cold and stirred for one hour, after which it was transferred to a large beaker, neutralized with hydrochloric acid and evaporated to dryness under hot air from an electrically heated blower. The dried residue was refluxed with 300 ml. of ethyl acetate for 30 minutes. During refluxing, the mixture was stirred with a magnetic stirrer. After refluxing, the ethyl acetate solution was decanted to a beaker and cooled to facilitate crystallization of the acid. The crystals were washed first with cold ethyl acetate, then ether and air dried. In order to increase product yield and conserve ethyl acetate, the mother liquor from crystallization and ethyl acetate used for product washing was saved and used for subsequent reflux extraction.

Acetohydroxamic acid produced by this procedure was further characterized by comparing its urease inhibition with a known sample. Inhibition was studied in vitro using the method of Brent and Adepoju (1967). It was also characterized by the ferric ion-hydroxamic acid color reaction reported by Lipmann and Tuttle (1945).

Animals and Management. A pair of twin Hereford steers fitted with permanent rumen fistulae were used in the experiment. The animals were housed indoors in a dirt-floored pen bedded with shavings. Water was available ad libitum.

Experimental Diet. The concentrate portion of the experimental diet is shown in Table 1.

Table 1. Concentrate Composition

Ingredient	%
Fine ground corn	97.5
Feed grade urea	1.7
Ground limestone	0.3
Salt	0.5

The animals were fed 2.720 kg. of the concentrate mixture and 1.000 kg. of prairie hay per feeding. These feeding levels had been established as an amount of feed the animals would readily consume. The animals were tied until they had finished eating, to remove the need for individual housing.

Feed was supplied at 12 hour intervals, 7:00 a.m. and 7:00 p.m.

Proximate analysis of the feeds is shown in Table 2. The diet supplied 46.27 gm. urea per feeding (92.54 gm. per day).

Table 2. Proximate Composition of Feeds

	Concentrate	Hay	Total diet (calculate)
% Dry Matter	87.99	91.15	88.84
% Crude Protein	14.29	5.31	11.88
% Ether Extract	4.57	2.37	3.98
% Crude Fiber	2.95	31.35	10.58
% Nitrogen Free Extract	63.58	45.44	58.78
% Ash	2.60	6.41	3.62

Experimental Design. The study was designed to study (1) the effect of acetohydroxamic acid on ruminal ammonia levels *in vivo* and (2) possible residual effects of acetohydroxamic acid. Thus, following adjustment of the steers to their surroundings and the establishment of their ration consumption, the experiment was carried out using the schedule in Table 3.

Table 3. Experimental Design

Treatment	Length	Dates
Control Period 1	6 days	April 7 to April 12
3 gm. AHA/feeding	6 days	April 13 to April 18
Control Period 2	6 days	April 19 to April 24
6 gm. AHA/feeding	6 days	April 25 to April 30
Control Period 3	6 days	May 1 to May 6

Sampling. Samples were drawn through metal strainers suspended in the rumen by rubber hoses. The hoses passed through the plastic caps of the cannulae and were sealed by pinch clamps. Samples were drawn by using a large metal syringe. Rumen fluid was drawn up and ejected through the tubes and strainers several times to insure that the samples represented rumen contents and not fluid remaining in the system from the previous sampling.

Samples were removed from the rumen according to Table 4.

Table 4. Sampling Schedule

Time	Hours after Feeding	
7:00 a.m.	0	(Sample taken just before feeding)
8:00 a.m.	1	
9:00 a.m.	2	
10:00 a.m.	3	
11:00 a.m.	4	
1:00 p.m.	6	
3:00 p.m.	8	
5:00 p.m.	10	
7:00 p.m.	12	

They were strained through 4 layers of cheesecloth, mixed with 0.5 ml. of 50% sulfuric acid and stored in test tubes for subsequent analysis.

Analysis. Rumen ammonia was determined by the method of Conway (1963). About 1 ml. of a 0.5% boric acid solution containing a mixed indicator of methyl red and bromocresol green was placed in the center well of a Conway dish.[1] About 1 ml. of 45% sodium carbonate solution was added to the sealing ring. The sample (0.5 ml. rumen fluid) was added to the sample ring opposite the sodium carbonate. The lid was set in place, turned to form a seal, and the dish gently

[1]Conway dish: Biological Research, Inc., St. Louis 21, Missouri.

rotated to mix the rumen fluid with the carbonate. The ammonia evolved from the alkaline solution was absorbed in the boric acid, changing the indicators to their salt form. Following one hour of incubation, the contents of the center well were titrated to the original color with 0.1 N sulfuric acid, using a micrometer burette[2] and a 2 mm. x 10 mm. magnetic stirring bar. The procedure was standardized against ammonia standards with 50, 100, and 200 mcg./ml. ammonia nitrogen. Duplicate analyses were run on all samples. The procedure and calculation are explained in more detail by Brent (1967).

Statistical Analysis. Analyses were carried out with an IBM 360 computer by analysis of variance (Snedecor and Cochran, 1967).

The design of the experiment allowed partitioning of variance due to treatments, steers, days and hours. All possible 2-way and 3-way interactions were computed. The mean square for the single 4-way interaction was used as the error term. Differences between individual means was determined by Duncan's Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Data obtained from the experiment are shown in Fig. 1. The rapid increase in ammonia nitrogen soon after feeding, followed by a decline, is fairly typical of high urea diets.

---

[2]Syringe Microburet: Micro-metric Instrument Co., Cleveland, Ohio.

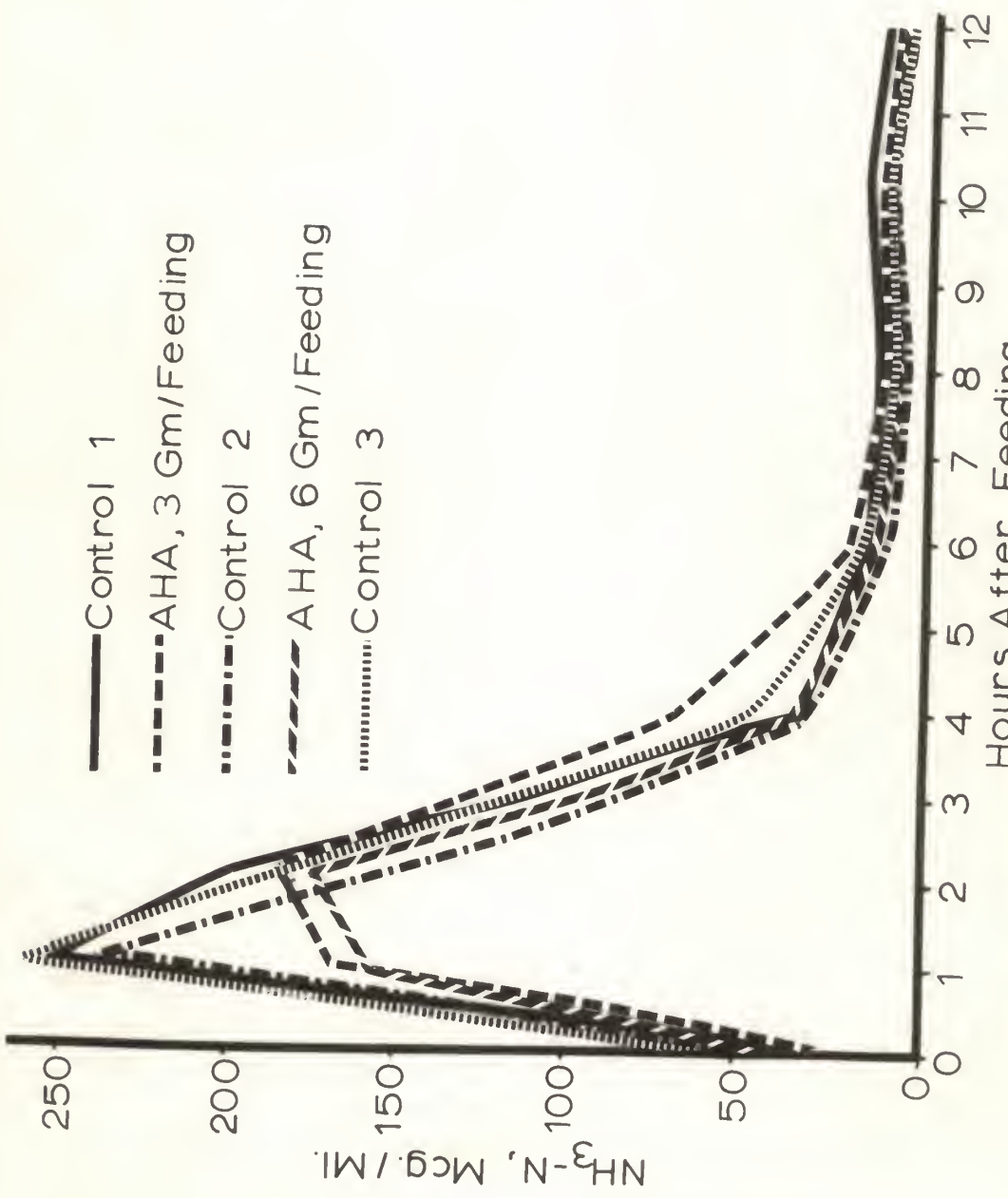

Fig. 1. Rumen ammonia levels with time after feeding.

Nitrogen is lost to the rumen economy in the first few hours after feeding by diffusion across the rumen wall. The ammonia levels during the final six hours may be sufficiently low that the microorganisms are, in effect, deficient in nitrogen.

Analysis of variance data for the experiment is shown in Table 5. The analysis indicates a significant ($P < .001$) difference in mean ammonia levels between the experimental periods. Applications of Duncan's (1955) Multiple Range Test to these means (Appendix Table 1) shows that there were no significant differences in rumen ammonia between the first and last control periods (periods 1 and 5). However, the second control period (period 3) yielded ammonia levels which were lower ($P < .05$) than the other two control periods, and application of Duncan's (1955) Multiple Range Test to two-way interaction means for treatment and hours (Appendix Table 2) shows that acetohydroxamic acid at both 3 and 6 gm. per feeding depressed rumen ammonia ($P < .05$) at one hour after feeding. Although there are significant ($P < .05$) differences in two-way interaction means at 2, 3, and 4 hours after feeding, the differences follow no logical pattern and consequently, their biological consequence is doubtful. Differences at hours 6, 8, 10, and 12 were non-significant. Duncan's Multiple Range Test (1955) shows a lower mean rumen ammonia value for 6 gm. acetohydroxamic acid versus 3 gm. ($P < .01$) (Appendix Table 1). However, differences in 12-hour rumen ammonia means may have very limited biological importance.

Table 5. Analysis of Variance

Source of Variance	D.F.	M.S.	F.	Significance
Period	4	6032.34	17.87	$P < .001$
Day	5	519.27	1.54	ns
Period x Day	20	1342.32	3.98	$P < 0.001$
Steer	1	1782.88	5.28	$P < 0.05$
Period x Steer	4	3136.93	9.29	$P < 0.001$
Day x Steer	5	789.77	2.34	$P < 0.05$
Period x Day x Steer	20	673.61	2.00	$P < 0.05$
Hour	8	384680.08	1139.34	$P < 0.001$
Period x Hour	32	3815.56	11.30	$P < 0.001$
Day x Hour	40	337.67	1.00	ns
Period x Day x Hour	160	712.43	2.11	$P < 0.001$
Steer x Hour	8	346.33	1.03	ns
Period x Steer x Hour	32	651.93	1.93	$P < 0.01$
Day x Steer x Hour	40	329.34	0.98	ns
Period x Day x Steer x Hour	160	337.63		

Ammonia loss from the rumen is more closely related to levels shortly after feeding. Since ammonia levels are quite similar at hours 1 and 2 on the two levels of acetohydroxamic acid, the usefulness of more than 3 gm. per feeding appears questionable.

In vitro results (Brent and Adepoju, 1967) indicate that urease is very sensitive to inhibition by acetohydroxamic acid. In their work, 20 mcg. acetohydroxamic acid per ml. rumen fluid inhibited rumen urease (in vitro) 50%. If one assumes the rumen volume of the steers on the present study to be about 120 l., then the initial concentration of acetohydroxamic acid should approximate 25 and 50 mcg. per ml. for the 3 gm. and 6 gm. per feeding treatments respectively. Since urease inhibition on these treatments was considerably less than the 50% observed in vitro, and since inhibition essentially disappeared by two hours after feeding, it appears that either the urease concentration in vivo is much greater, or the inhibitor passes rapidly through the rumen wall. Baintner (1964b) observed acetohydroxamic acid in the urine of sheep very shortly after administration, indicating the latter explanation may be true.

Table 5 indicates a significant ($P < .05$) difference between supposedly identical twin steers. Although enzymatic pathways in the somatic tissue and absorption rates from the gastrointestinal tract may be quite similar, large differences may exist in the rumen fermentation. Thus, the

validity of rumen fermentation data obtained from identical steers may be open to question.

Table 5 indicates that there were no differences in mean rumen ammonia values attributable to days. This lends further proof that acetohydroxamic acid exerts no cumulative or residual effects, and indicates that shorter studies on this compound should be statistically valid.

Future work needs to be directed toward slowing the rate of removal of acetohydroxamic acid from the rumen. The ruminal metabolism of this compound needs to be more fully understood. Both of these questions could be studied by the use of ^{14}C-acetohydroxamic acid. Use of this material could also allow examination of excretion pathways and tissue residue.

Acetohydroxamic acid will complex a number of metals. Absorption of metal complexes of acetohydroxamic acid through the rumen mucosa may be different than acetohydroxamic acid itself. These complexes may offer a method for lengthening the stay of acetohydroxamic acid in the rumen.

Research is needed in rapid, and economical production of acetohydroxamic acid. It seems unlikely that feedlot studies can be carried out with the compound as long as it must be produced in small laboratory quantities.

Ammonia levels at one hour after feeding appear to be the most sensitive indicator of ruminal urease activity. With this in mind, means of three way interactions for days,

treatments and hours were examined for data one hour after feeding (Appendix Table 3). Although significant ($P < .05$) differences exist in these data, no ascending or descending pattern was observed in control or treatment periods. Thus, acetohydroxamic acid, at least under the condition of this study, appears to have neither cumulative nor residual effects.

Acetohydroxamic acid is one of the first compounds enabling the rumen nutritionist to manipulate ammonia levels in the rumen. This fact in itself should justify increased research in the compound with an eye toward improving non-protein nitrogen utilization in the ruminant.

SUMMARY

This experiment was designed to study the in vivo inhibition of rumen urease, using two different levels of acetohydroxamic acid (3 gm. or and 6 gm. per feeding), and also to ascertain if this compound has cumulative and/or residual effects.

Fistulated twin Hereford calves maintained indoors and bedded with shavings were given either 3 or 6 gm. of acetohydroxamic acid at feeding time, dissolved in water and administered through the rumen fistula, according to the following schedule:

1. Control Period 1 — 6 days
2. 3 gm. Acetohydroxamic acid/feeding — 6 days
3. Control Period 2 — 6 days

4. 6 gm. Acetohydroxamic acid/feeding 6 days

5. Control Period 3 6 days

Rumen ammonia levels were measured by the Conway micro-diffusion technique.

Analysis of variance shows a significant ($P < .001$) difference in mean ammonia levels between the experimental periods. Application of Duncan's (1955) Multiple Range Test to these means shows that there were no significant differences in rumen ammonia levels between the first and last periods. In the second control period (period 3) the ammonia levels were lower than in the other two control periods. Mean rumen ammonia was lower ($P < .05$) with 6 gm. acetohydroxamic acid than with 3 gm.

Acetohydroxamic acid at both 3 gm. and 6 gm. per feeding diminished the peaks of rumen ammonia during the first hour after feeding. In the second, third, and fourth hour after feeding there were significant ($P < .05$) differences in rumen ammonia, but no relationship between inhibitor and ammonia was observed. No differences were seen in the other hours. It was observed that the levels of ammonia at one hour after feeding were not statistically different with the two levels of inhibitor and is possible that the use of 6 gm. of acetohydroxamic acid may not be useful due to rapid movement of the inhibitor out of the rumen. More research must be conducted to better understand the metabolism of this compound in ruminants and to work out a way that its rate of

loss from the rumen may be lowered. Ideally, an inhibitor level should be used that would decrease ammonia levels in the first few hours after feeding, and lead to higher ammonia levels later in the day. In this way the concentration of nitrogen available for bacterial protein synthesis would more closely approximate the synthetic capabilities of the rumen population.

The data show that residual and cumulative effects from acetohydroxamic acid are unlikely. Certainly it is rapidly lost from the rumen. Thus, inhibitory effect is of short duration. High levels of rumen ammonia on the first day of the last control period (after the 6 gm. period) give further evidence that no residual effects exist. During acetohydroxamic acid treatments, ammonia levels on consecutive days showed no consistent pattern. Thus, cumulative effects due to the inhibitor are unlikely.

REFERENCES

Abe, Y. 1960. Sulfones. IV. The anti-bacterial activity *in vitro* and urine excretion of experimental animal. Osaka Shiritsu Daigaku Igaku Zashi. 9:4029. Chem. Abstr. 61:9977a.

Alkiewicz, J., Z. Eckestein, H. Halweg, P. Krakouka and T. Urbanski. 1957. Fungistatic activity of some hydroxamic acid. Nature. 180:1204.

Akkada, A. R. A. and T. H. Blackburn. 1963. Some observations on the nitrogen metabolism of rumen proteolytic bacteria. J. Gen. Microbiol. 31:461.

Arct, J., E. Czerwinska, Z. Eckestein, A. Ejmocki, R. Kowalik, W. Sobstka and E. Zukowski. 1964. Synthesis and fungicidal activity of aryloxyalkanehydroxamic acids and analogs. Przeniysl Chem. 43:332.

Ayoga, T., and W. H. J. Summerskill. 1966. Inhibition by acetohydroxamic acid of human mucosal and faecal urease-specific activity. Lancet. 7432:296.

Bahadur, K. and V. Chandra. 1959. Decomposition of urea by urease. Enzymologia. 21:1.

Bahadur, K. and V. Chandra. 1960. Influence of stannous chloride on the decomposition of urea by soybean, glycin soja (G-mas.), urease. Enzymologia. 22:60.

Baintner, K., Jr. 1964a. Prevention of urea poisoning with acetohydroxamic acid. Allattenyesz 13:373. Chem. Abstr. 63:3531e.

Baintner, K., Jr. 1964b. Inhibition of the activity of rumen urease *in vitro*. Allatteyesz 57:17. Chem. Abstr. 65:5770c.

Baldwin, E. 1967. *Dynamics Aspects of Biochemistry*. Cambridge University Press.

Bernheim, M. L. C. 1964. The enzymatic hydrolysis of certain hydroxamic acids. Arch. Biochem. Biophys. 107:313.

Bernheim, M. L. C. 1965. Metabolism of anthranilic hydroxamic acid by rat liver. Arch. Biochem. Biophys. 112:191.

Brent, B. E. and D. Richardson. 1967. The apparent intracellular nature of rumen urease. J. Animal Sci. 26:914. (Abstr.)

Brent, B. E. and A. Adepoju. 1967. Effect of acetohydroxamic acid on rumen urease. J. Animal Sci. 26:1428. (Abstr.)

Brent, B. E. 1967. Ammonia by microdiffusion analysis. Unpublished. Mimeograph. Department of Animal Husbandry, Kansas State University.

Caradonna, E. and M. L. Stein. 1960. Synthesis and bacteriostatic activity of hydroxamic acid derivatives of isoxazole. Il Farmaco. 15:647.

Chalupa, W., J. H. Clark, P. Opliger and R. Lavker. 1968a. Enzyme systems for ammonia metabolism in the rumen. Federation Proceedings. 27:557. (Abstr.)

Chalupa, W., J. H. Clark, P. Opliger and R. Lavker. 1968b. Enzyme systems for ammonia metabolism in ruminant liver. J. Dairy Sci. 51:979. (Abstr.)

Clifford, A. J., J. R. Bourdette and A. D. Tillman. 1968. Studies on ruminal urease activity. J. Animal Sci. 27:814.

Conway, E. J. 1957. Microdiffusion Analysis and Volumetric Error. 4th ed. Crosby Lockwood and Sons, Ltd., London.

Coutts, R. T., D. Noble and D. G. Wibberly. 1964. Some cyclic hydroxamic acids. J. Pharm. Pharmacol. 16:773.

Coutts, R. T., W. N. Pitkethley and D. G. Wibberly. 1965. Antibacterial activity of some quinolines containing a cyclic hydroxamic acid group. J. Pharm. Sci. 54:792.

Coutts, R. T. and K. W. Hindmarsh. 1966. 2-H-1-4-benzoxazine hydroxamic acids and related compounds. Can. J. Pharm. Sci. 1:11.

Coutts, R. T. 1967. Hydroxamic acids. I. Acyclic hydroxamic acids. Can. J. Pharm. Sci. 2:1.

Duncan, D. B. 1955. Multiple Range and Multiple F Test. Biometrics. 11:1.

Eckstein, Z. and T. Urbanski. 1956. Fungicide activities of some aryloacetohydroxamic acids. Bull. Acad. Polon. Sci. 4:627. Chem. Abstr. 51:8695a.

Eckstein, Z. and E. Czerwinskafejgin. 1959. The properties and fungicidal activity of aryloxy-acetohydroxamic acids. Przemsysl. Chem. 38:213. Chem. Abstr. 61:77990.

Eckstein, Z. and H. Domanoka. 1965. Properties systemiques des derives des acides hydroxamiques. Medel Landbonwhogesch Opzoekingssta Staat Gent. 30:2007. Chem. Abstr. 67:19965.

Emery, T. and J. B. Neilands. 1959. The iron-binding center of ferrichrome compounds. Nature. 184:1632.

Epstein, M. A., G. Freeman, L. S. D.'Agrosa and Louis Dulz. 1956. Toxicity of hydroxamic acid analogs. Prophylactic and therapeutic efficacy against nerve gas poisoning in mice. Fed. Proc. Exp. Biol. Med. 92:660.

Fasman, G. D. and C. Nieman. 1951. A reinvestigation of the kinetics of the urease-catalyzed hydrolysis of urea. I. The activity of urease in the presence of sodium and potassium phosphate. J. Am. Chem. Soc. 73:1646.

Fedorova, L. S. 1965. The bond of urease with the bacterial cell. Zh. Mikrobiol. Epidemiol. i Immunobiol. 42:23. Chem. Abstr. 64:5474.

Fishbein, W. N. and P. P. Carbone. 1963. Hydroxyurea: mechanism of action. Science. 142:1069.

Fishbein, W. N., P. P. Carbone and D. H. Hochstein. 1965a. Acetohydroxamate; bacterial urease inhibitor with therapeutic potential in hyperammonaemic states. Nature. 208:46.

Fishbein, W. N., T. S. Winter and J. D. Davidson. 1965b. Urease catalysis I. Stoichiometry, specificity and kinetics of a second substrate: uydroxyurea. J. Biol. Chem. 240:2402.

Fishbein, W. N. 1967. Urease: A three substrate enzyme. Fed. Proc. 26:578. (Abstr.)

Fishbein, W. N. and C. L. Streeter. 1968. Acetohydroxamate-1 ^{14}C: Physiologic disposition in the mouse. Fed. Proc. 27:238. (Abstr.)

Gale, G. R. 1966. Hydroxyurea derivatives: Oxamylhydroxamic acid. Cancer Res. 26:2340.

Gale, G. R. and J. B. Hynes. 1966. Further studies of the antimicrobacterial agents glycylhydroxamic acid and B-alanyllhydroxamic acid. Can. J. Microbiol. 12:73.

Gibbons, R. J. and R. D. McCarthy. 1957. Obligately anaerobic urea-hydrolizing bacteria in the bovine rumen. J. Dairy Sci. 40:635. (Abstr.)

Gibbons, R. J. and R. N. Doetsch. 1959. Physiological study of an obligately anaerobic ureolytic bacterium. J. Bact. 77:417.

Glimp, H. A. and A. D. Tillman. 1965. Effect of jackbean urease injections on performance, anti-urease production, and plasma ammonia and urea levels in sheep. J. Animal Sci. 24:105.

Gorin, G., E. Fuchs, L. G. Butter, S. L. Chopra and R. T. Hersh. 1962. Properties of urease. Biochem. 1:911.

Gorin, G. and C. C. Chin. 1965. Urease IV. Its reaction with N-ethylmaleimide and with silver ion. Biochem. Biophys. Acta. 99:418.

Halweg, H. and P. Krakouka. 1956. Fungistatic effect of some aromatic hydroxamic acids. Gruzlica 24:233.

Harbers, L. H., A. D. Tillman and W. J. Visek. 1962. Effect of barbituric acid on urea diets for ruminants. J. Animal Sci. 21:754.

Harman, K. M. and C. Nieman. 1949. The competitive inhibition of the urease-catalyzed hydrolysis of urea by phosphate. J. Biol. Chem. 177:601.

Hase, J. and K. Kobashi. 1967. Inhibition of Proteus vulgaris urease by hydroxamic acids. J. Biochem. 62:293.

Hellerman, L., F. P. Chinard and V. R. Deitz. 1943. Protein sulfhydryl group and the reversible inactivation of the enzyme urease. J. Biol. Chem. 147:443.

Henderick, H. 1962. Ureolytic activity of rumen micro-organisms. Medelel. Landbonwhogeschool Opzoekingsta Staat Gent. 27:1481. Chem. Abstr. 60:4565.

Johnson, R. R., O. G. Bentley and T. V. Hershberger. 1962. The effectiveness of control urea prills and copper-treated urea prills as nitrogen sources for rumen micro-organisms. Ohio Agr. Exp. Sta. Res. Bul. 917.

Jones, G. A., R. A. McLeod and A. C. Blackwood. 1964. Ureolytic rumen bacteria. I. Characteristics of the microflora from a urea-fed sheep. Can. J. Microbiol. 10:371.

Kobashi, K., J. Hase and K. Uehara. 1962. Specific inhibition of urease by hydroxamic acids. Biochem. Biophys. Acta. 65:383.

Kobashi, K., J. Hase and T. Komai. 1966. Evidence for the formation of an inactive urease-hydroxamic acid complex. Biochem. Biophys. Res. Commun. 23:34.

Lambelin, G., N. P. Buu-Hoi, G. Mees, J. Thiriaux and M. Huriaux. 1966. Pharmacological study of p-butoxyphenylacethyl-hydroxamic acid. II. Toxicological research. Med. Pharmacol. Exp. 15:545. Chem. Abstr. 66:9818q.

Larson, A. D. and R. E. Kallio. 1954. Purification and properties of bacterial Urease. J. Bact. 68:67.

Lattimer, J. K., H. Seneca, H. H. Zinsser and J. T. Donovan. 1961. Drug-resistant bacteria made drug-suxceptible by enzyme inhibitors. J. Am. Med. Assoc. 78:764.

Levenson, S. M., L. V. Crowley, R. E. Harowitz and O. J. Malm. 1959. Metabolism of carbon labeled urea in the germ free rat. J. Biol. Chem. 234:2061.

Lipmann, F., and L. C. Tuttle. 1945. A specific micromethod for the determination of acetylphosphate. J. Biol. Chem. 159:21.

Loper, D. C., L. H. Harbers and D. Richardson. 1967a. Evaluation of Ruminal urease inhibitors *in vitro*. J. Animal Sci. 26:924. (Abstr.)

Loper, D. C., L. H. Harbers and D. Richardson. 1967b. Effect of bacitracin-MD, Copper Sulfate and neomycin sulfate on ruminal urease *in vivo*. J. Animal Sci. 26:924. (Abstr.)

Loper, D. C. 1967. Evaluation of ruminal urease inhibitors. Ph. D. thesis, Kansas State University.

Lyubimov, V. I. 1955. The splitting of urea in bacteria as an intracellular process. Mikrobiol. 24:160. Chem. Abstr. 49:106067.

McKay, E. S. M. and A. E. Oxford. 1954. Some facultatively anaerobic Gram-negative rods from the rumen of the calf and the sheep. J. Gen. Microbiol. 11:472.

Mostafa, I. Y., S. M. A. D. Zayed and M. Farghaly. 1966. Fungicidal properties of thioacetohydroxamic acids and related compounds. Ark. Microbiol. 55:324.

Muhrer, M. E. and E. J. Carrol. 1964. Urea utilizing microorganisms in the rumen. J. Animal Sci. 23:885. (Abstr.)

Neilands, J. B. 1967. Hydroxamic acids in nature. Science. 156:1443.

Noble, D. and D. G. Wibberly. 1966. Some anti-bacterial quinolines containing a cyclic hydroxamic acid group. J. Med. Chem. 9:974.

Perlzweig, W. A. 1932. The activation of urease. Science. 76:435.

Pianotti, R. S., R. R. Mohan and B. S. Schwartz. 1966. Proteus vulgaris urease: in vitro inhibition by urea analogs. Proc. Soc. Exp. Biol. Med. 122:506.

Roncucci, R., M. J. Simon, G. Lambelin, N. P. Buu-Hoi and J. Thiriaux. 1966. Distribution, metabolism and excretion of p-butoxyphenylacetohydroxamic acid, an anti-inflamatory agent. Biochem. Pharmacol. 15:1563.

Seneca, H., J. K. Lattimer, P. Peer and P. F. Stuart. 1961. Urease activity of some lysates of pathogenic bacteria. Lancet. 1:1166.

Seneca, H., P. Peer and R. Nally. 1962. Microbial urease. Nature. 193:1106.

Shaw, W. R. 1953. The inhibition of urease by various metal ions. J. Am. Chem. Soc. 76:2160.

Shimura, K., M. Saejima and E. Kikuchi. 1958. Mechanism of urea utilization by the ruminant. I. Urease in beef gastric mucosa. J. Agr. Chem. Soc. 32:913.

Snedecor, G. W. and W. G. Cochran. 1967. Statistical Methods. 6th ed. The Iowa State College Press, Ames, Iowa.

Sumner, J. B. 1926. The isolation and crystallization of the enzyme urease. J. Biol. Chem. 69:435.

Sumner, J. B. and D. B. Hand. 1928. Crystalline urease. J. Biol. Chem. 76:149.

Sumner, J. B. and G. F. Somers. 1953. Chemistry and Methods of Enzymes. 3rd. ed. Academic Press.

Utley, J. D. 1963. The effect of anthranilic hydroxamic acid in rat behavior and rat brain gamma-aminobutyric acid, norepinephrine and 5-hydroxytriptamine concentration. J. Neurochem. 10L423.

Venulet, J., K. Jakimouska and A. Urbanska. 1956. The behavior of hydroxamic acids in animal organisms. Bull. Acad. Polon. Sci. 4:189. Chem. Abstr. 51:14108d.

Wang, J. H. and D. A. Tarr. 1955. On the mechanism of urease action. J. Am. Chem. Soc. 77:6205.

APPENDIX

Appendix Table 1. Rumen Ammonia Means by Treatments

Treatment	No. Observation	Mean (mcg. NH_3-N/ml.)
1, Control Period 1	108	77.71a*
2, 3 gm. AHA/Feeding	108	70.86b
3, Control Period 2	108	65.38c
4, 6 gm. AHA/Feeding	108	59.92d
5, Control Period 3	108	76.34a

*(P .05).

Appendix Table 2. Two-way Interaction Means For Treatment and Hour (CG.)

Factors		No. Observation	Mean
Treatment*	Hour		mcg. NH_3-N/ml.
1	0	12	31.98
1	1	12	249.99
1	2	12	202.77
1	3	12	109.86
1	4	12	37.69
1	6	12	17.73
1	8	12	14.94
1	10	12	20.48
1	12	12	13.97
2	0	12	31.70
2	1	12	168.13
2	2	12	181.73
2	3	12	131.32
2	4	12	65.55
2	6	12	21.11
2	8	12	12.85
2	9	12	14.85
2	12	12	10.93
3	0	12	22.60
3	1	12	237.38
3	2	12	179.62
3	3	12	81.96

Appendix Table 2. (continued)

Factors		No. Observation	Mean
Treatment	Hour		mcg. NH_3-N/ml.
3	4	12	33.58
3	6	12	9.9
3	8	12	6.0
3	10	12	10.53
3	12	12	6.9
4	0	12	34.29
4	1	12	158.75
4	2	12	174.39
4	3	12	92.48
4	4	12	34.45
4	6	12	12.35
4	8	12	9.3
4	10	12	13.29
4	12	12	10.00
5	0	12	31.61
5	1	12	260.28
5	2	12	199.15
5	3	12	100.63
5	4	12	41.83
5	6	12	19.30
5	8	12	12.84
5	10	12	12.26
5	12	12	9.2

*See Appendix Table 1.

Appendix Table 3. Three-way Interaction Means For Period, Day and Hour One Hour after Feeding

	Day					
	1	2	3	4	5	6
	mcg. NH_3-N/ml.					
Control Period 1	274.4	243.4	249.4	215.3	263.0	255.6
3 gm. AHA/Feeding	175.5	138.2	209.2	132.5	200.0	153.5
Control Period 2	219.0	279.8	163.6	238.7	242.3	281.2
6 gm. AHA/Feeding	163.0	124.4	154.4	147.2	152.7	210.9
Control Period 3	312.7	281.5	267.7	253.7	284.2	161.9

Appendix Table 4. Average Hourly Rumen Ammonia Levels (CG.)

Control Period 1

Hour	A*	D**	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D
	1	1	2	1	1	2	2	2	1	3	2	3	1	4	2	4	1	5	2	5	1	6	2	6
0	23.8		27.6		26.1		23.7		19.3		26.8		35.9		27.2		62.3		37.8		37.9		35.4	
1	262.6		286.1		240.4		246.4		238.8		260.0		299.6		130.9		289.7		234.2		270.8		240.4	
2	209.3		201.7		227.2		182.1		195.7		229.6		221.9		158.6		216.1		186.7		205.7		198.6	
3	86.5		84.4		124.1		77.5		121.3		108.9		115.9		119.6		136.9		124.4		115.1		103.9	
4	37.2		19.7		54.4		12.5		37.2		32.1		44.2		65.7		47.9		26.6		50.8		24.0	
6	21.3		11.9		21.8		20.1		33.6		11.2		13.2		20.2		17.6		12.4		23.9		5.5	
8	25.0		17.5		10.0		9.1		21.1		4.3		18.2		19.4		22.3		4.4		17.3		10.7	
10	25.8		22.6		32.2		19.5		20.6		7.3		21.9		17.2		23.9		20.1		19.5		15.2	
12	17.8		11.9		8.4		15.4		10.6		11.8		17.2		13.7		20.3		10.0		16.5		14.0	

3 gm. AHA/Feeding

Hour	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D
	1	1	2	1	1	2	2	2	1	3	2	3	1	4	2	4	1	5	2	5	1	6	2	6
0	38.8		21.7		39.8		23.1		32.0		35.3		39.7		27.8		33.4		24.6		36.9		27.3	
1	195.1		155.9		169.6		107.8		251.2		167.1		196.7		68.3		213.5		186.4		169.3		137.7	
2	173.1		164.6		195.6		161.4		189.2		199.1		202.8		203.3		189.5		178.2		168.6		155.4	
3	210.8		175.3		99.6		136.2		102.3		132.3		185.0		128.7		125.9		125.7		79.0		75.1	
4	112.4		82.3		47.4		83.2		50.1		65.0		87.9		71.8		45.5		61.1		52.5		27.4	
6	43.4		25.0		19.4		29.2		3.9		15.7		22.8		30.0		15.1		21.0		14.5		13.3	
8	24.1		18.0		21.7		4.5		11.0		4.2		23.0		10.6		17.0		10.0		5.9		4.2	
10	40.4		23.3		20.5		8.5		7.9		9.7		14.0		4.6		11.3		9.5		16.4		7.3	
12	13.7		8.8		13.8		8.5		13.4		11.0		17.1		8.5		9.9		6.5		7.1		12.8	

*Animal.

**Day within treatment.

Appendix Table 4. (continued)

Control Period 2

Hour	A*	D**	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D
	1	1	2	1	1	2	2	2	1	3	2	3	1	4	2	4	1	5	2	5	1	6	2	6
0	15.7		17.5		25.5		24.8		15.4		15.0		28.3		21.3		21.4		23.7		32.8		29.8	
1	178.4		259.5		264.0		293.6		154.4		172.8		243.2		234.2		228.7		255.9		298.4		265.4	
2	176.3		199.3		192.9		217.7		113.6		124.4		167.9		214.3		160.5		183.2		193.6		211.7	
3	93.0		75.8		87.8		103.0		53.6		58.5		93.8		99.8		83.1		99.8		109.0		119.0	
4	39.8		28.8		32.2		42.5		12.2		17.2		24.3		36.9		34.1		47.2		34.2		53.6	
6	15.9		3.4		26.1		11.9		5.0		2.8		6.5		8.3		6.3		12.5		7.7		11.9	
8	15.0		6.6		8.8		3.3		4.2		3.1		6.8		4.2		5.0		1.7		10.4		3.1	
10	12.2		14.5		9.7		12.9		9.6		8.1		8.0		13.8		3.9		2.9		16.5		14.3	
12	5.6		5.1		3.3		4.9		7.5		4.8		8.6		8.4		7.2		4.1		11.1		12.4	

6 gm. AHA/Feeding

Hour	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D
	1	1	2	1	1	2	2	2	1	3	2	3	1	4	2	4	1	5	2	5	1	6	2	6
0	34.8		38.5		25.7		22.6		69.1		22.8		51.0		32.3		27.9		23.0		33.4		30.4	
1	172.4		153.7		155.2		93.6		184.9		123.9		162.6		131.7		151.6		153.7		220.2		201.5	
2	166.6		174.0		137.2		120.5		188.3		174.1		182.4		163.3		172.9		170.4		236.8		206.2	
3	85.0		94.8		50.0		87.3		129.6		103.8		111.7		95.3		77.3		67.2		106.7		101.1	
4	27.9		28.9		11.0		8.3		69.1		45.0		45.1		43.3		40.7		29.8		27.4		36.9	
6	12.7		4.6		14.1		4.0		45.0		8.5		14.9		8.3		7.6		7.8		8.6		12.1	
8	8.8		5.2		20.8		3.8		14.5		4.7		7.3		4.6		19.0		6.8		6.8		8.9	
10	18.2		13.6		29.5		8.8		13.5		6.1		16.6		8.6		9.7		23.0		2.4		9.5	
12	8.8		10.1		14.8		7.0		12.5		5.4		6.8		6.9		17.0		13.0		7.0		10.3	

*Animal.

**Day within treatment.

Appendix Table 4. (continued)

	Control Period 3																							
Hour	A*	D**	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D
	1	1	2	1	1	2	2	2	1	3	2	3	1	4	2	4	1	5	2	5	1	6	2	6
0	33.3		24.5		33.1		19.5		26.9		37.9		33.6		34.9		32.5		29.0		34.4		39.7	
1	321.2		304.2		269.3		293.7		299.4		236.0		230.8		276.5		257.4		311.0		61.9		261.9	
2	187.3		220.4		217.2		237.4		166.8		228.1		247.6		189.0		199.2		186.1		96.9		213.8	
3	75.1		84.3		71.2		120.5		85.5		95.7		102.8		78.3		95.5		92.1		78.7		227.8	
4	21.7		25.7		27.7		40.2		28.9		39.9		31.1		27.7		33.3		25.1		61.9		138.8	
6	7.4		10.1		13.1		10.8		9.4		11.4		25.4		23.7		20.5		11.6		41.2		47.0	
8	15.8		9.6		11.3		13.6		12.9		4.0		15.1		8.2		14.0		9.1		32.2		8.3	
10	9.6		10.4		20.2		10.6		5.3		12.0		12.1		13.8		4.6		20.3		23.2		5.0	
12	7.6		8.2		18.1		10.4		8.4		13.4		6.0		10.1		4.1		11.7		8.1		3.8	

*Animal.

**Day within treatment.

INHIBITION OF RUMINAL UREASE *IN VIVO* BY ACETOHYDROXAMIC ACID

by

FABIO de BORJA PORTELA

B. S., University of Minas Gerais, Brazil, 1963

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Animal Husbandry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1968

This experiment was conducted in an attempt to examine closely the effects of two levels of acetohydroxamic acid, a urease inhibitor, on the rumen ammonia level and to ascertain whether or not acetohydroxamic acid has a residual and/or cumulative effect on urease activity after withdrawal of the inhibitor from the diet.

A pair of twin Hereford steers fitted with permanent rumen fistulae was housed indoors in a dirt-floored pen bedded with shavings. Water was available ad libitum. The animals were fed a concentrate mixture (2.720 kg.) and prairie hay (1 kg.) twice daily. The concentrate mixture contributed 46.24 gm. urea per feeding.

Two 6-day treatment periods, in which both steers received either 3 or 6 gm. acetohydroxamic acid were preceeded, separated, and followed by three 6-day control periods. This allowed for observations on residual and cumulative effects of both levels of acetohydroxamic acid.

Rumen samples were obtained 0, 1, 2, 3, 4, 6, 8, 10 and 12 hours after feeding, strained through four layers of cheesecloth, and preserved with sulfuric acid for subsequent ammonia analysis by the Conway microdiffusion technique.

Analysis of variance showed significant ($P < .001$) differences between treatment periods and between control periods, when comparing over-all means. Examination of two-way interaction for periods and hours showed that at one hour after feeding, ammonia was higher for the three control

periods than for the two treatment periods ($P < .05$). The ammonia levels at one hour after feeding for the two treatments were not significantly different. Ammonia levels during the 6-day treatment and control periods followed no consistent ascending or descending pattern due to days, indicating that acetohydroxamic acid probably exerts neither cumulative nor residual effects on rumen urease. Differences in urease inhibition between the 3 and 6 gm. inhibitor levels appear questionable. Acetohydroxamic acid at both levels delayed peak rumen ammonia appearance by one hour.